1: AJ000220. Mus musculus mRNA...[gi:2370296]

NEW Links

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DEFINITION  Mus musculus mRNA for estrogen receptor subtype beta.
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VERSION     AJ000220.1  GI:2370296
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SOURCE      Mus musculus.
  ORGANISM  Mus musculus
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            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1458)
  AUTHORS   Pettersson,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-JUL-1997) Pettersson K., Department of Medical
            Nutrition, Karolinska Institute, F 60 Novum, S-141 86 Huddinge,
            SWEDEN
REFERENCE   2  (bases 1 to 1458)
  AUTHORS   Pettersson,K., Grandien,K., Kuiper,G.G. and Gustafsson,J.A.
  TITLE     The mouse estrogen receptor beta form estrogen response element
            binding heterodimers with the estrogen receptor alpha
  JOURNAL   Unpublished
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Mouse Estrogen Receptor β Forms Estrogen Response Element-Binding Heterodimers with Estrogen Receptor α

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The recent discovery that an additional estrogen receptor subtype is present in various rat tissues has advanced our understanding of the mechanisms underlying estrogen signaling. Here we report on the cloning of the cDNA encoding the mouse homolog of estrogen receptor-β (ERβ) and the functional characterization of mouse ERβ protein. ERβ is shown to have overlapping DNA-binding specificity with that of the estrogen receptor-α (ERα) and activates transcription of reporter gene constructs containing estrogen-response elements in transient transfections in response to estradiol. Using a mammalian two-hybrid system, the formation of heterodimers of the ERβ and ERα subtypes was demonstrated. Furthermore, ERβ and ERα form heterodimeric complexes with retained DNA-binding ability and specificity *in vitro*. In addition, DNA binding by the ERβ/ERα heterodimer appears to be dependent on both subtype proteins. Taken together these results suggest the existence of two previously unrecognized pathways of estrogen signaling; I, via ERβ in cells exclusively expressing this subtype, and II, via the formation of heterodimers in cells expressing both receptor subtypes. (Molecular Endocrinology 11: 1486–1496, 1997)

INTRODUCTION

Nuclear receptors represent a large family of transcription factors that regulate the activity of target genes by direct binding to specific DNA recognition elements located in the vicinity of the transcription start site of genes. Members of this gene family have an evolutionary and functionally conserved structure with a hypervariable N-terminal region that contributes to the transactivation function, a centrally located, well conserved DNA-binding domain (DBD), and a C-terminal domain involved in ligand binding, dimerization and transactivation functions (1).

Steroid hormone receptors constitute a distinct subgroup within the nuclear receptor family (2), which includes receptors for glucocorticoids, mineralocorticoids, androgens, progestins, and estrogens [glucocorticoid receptor, mineralocorticoid receptor, androgen receptor, progesterone receptor, and estrogen receptor (ER), respectively]. In addition two orphan nuclear receptors, the ERR-1 and 2 (ER-related receptors) (3) have been referred to this group (4). The steroid hormone receptors bind as homodimers to palindromic DNA response elements (2). Another important feature of steroid hormone receptors is the interaction with the molecular chaperone hsp 90 (5).

Estrogens influence growth, differentiation, and function of many target tissues, including tissues of the female and male reproductive tract (6). Estrogens also play an important role in the maintenance of bone mass and in the cardiovascular system where estrogens have certain protective effects (7, 8). The ER-encoding cDNAs have been cloned from several species (9–12). Important examples of genes regulated by estrogens are the PR, epidermal growth factor receptor, certain growth factors (insulin-like growth factor-I, transforming growth factor-α and -β) and several protooncogenes (c-*fos*, c-*myc*, c-*jun*) (13). Loss of ER function has long been postulated to be incompatible with life, and therefore the successful generation of ER-deficient mice came as a surprise (14). These mice are viable but display severe dysfunction of the reproductive organs, and both sexes are sterile. The females have hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea. The fact that disruption of the ER gene did not completely eliminate the ability of small follicles to grow, as was evident from the presence of secondary and antral follicles in the knock-out mouse ovary, pointed to the possible existence of alternative ER mediating the intraovarian effects of estradiol. In some tissues from the ER knockout mice residual binding of estradiol with an affinity and specificity reminiscent of an ER protein could be

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Molecular Endocrinology

measured (14, 15). We have recently cloned a novel ER cDNA from rat prostate (16), which was suggested to be named rat ERβ subtype to distinguish it from the previously cloned ER cDNA (consequently ERα subtype). The rat ERβ protein was found to be highly homologous to the rat ERα protein, particularly in the DBD (>95% amino acid identity) and in the C-terminal ligand-binding domain (55% amino acid identity). In ligand-binding assays rat ERβ binds estrogens with an affinity and specificity resembling that of ERα, and ERβ is able to activate transcription of an estrogen-response element containing reporter gene construct (16, 17). In subsequent studies it was shown that ERβ is the primary ER subtype expressed in rat ovary and that ERβ message is down-regulated by gonadotropins in granulosa cells, suggesting that the functional significance of estrogen action in the rat ovary may be mediated primarily by ERβ (18).

The detailed biological significance of the existence of two ERs is presently unclear. Perhaps the existence of two ER subtypes may provide, at least in part, an explanation for the selective actions of estrogens and certain antiestrogens in different target tissues (19, 20).

In this paper we describe the cloning of the mouse ovary ERβ-cDNA and the characterization of the mouse ERβ protein with respect to DNA binding, homo- and heterodimerization, and transactivational functions. Finally, cotransfection of both ER subtypes with an estrogen response element (ERE) containing reporter gene construct showed that the formation of heterodimeric ERα/ERβ complexes may indeed constitute a novel estrogenic gene-regulatory pathway.

RESULTS AND DISCUSSION

A Homolog of Rat ERβ Is Expressed in Mouse Ovary

The recent discovery of a novel ER protein present in rat prostate and ovary (16) has given a new perspective to studies of estrogen action. The mouse represents an important model system for studies of gene function in mammals. We therefore investigated whether a homolog to the previously cloned rat ERβ (rERβ) is present in the mouse. Oligonucleotides, constructed to encompass the coding sequence of the rERβ cDNA, were used in an RT-PCR amplification of total RNA isolated from mouse ovaries. Amplification products with the expected size (~1.5 kbp) were subcloned and sequenced. The open reading frame of these clones displayed a high degree of amino acid identity with the rERβ protein and wer therefore r cognized as the mouse homolog of rERβ and will hereafter be referred to as the mouse ERβ (mERβ). As shown in Fig. 1, the mERβ amino acid sequence also manifests considerable similarities to mouse and rat ERα in the DNA- and ligand-binding domains (Fig. 1). Several amino acid residues that have been demonstrated to be required for high-affinity binding of estradiol by ERα (21) were found to be conserved in rat ERβ. The rat ERβ binds estradiol (E_2) with an affinity very comparable to that of ERα (17). Since the same amino acid residues are also conserved in the ligand-binding domain of mERβ, we concluded that mERβ should bind E_2 in a similar manner as the rat ERβ.

mERβ Protein Binds to an ERE

Members of the nuclear receptor superfamily bind to specific DNA motifs, generally palindromic or direct repeats of the sequence AG(A/G)(A/T)CA (22) located in the vicinity of the promoter of target genes. The specificity of DNA recognition by nuclear receptors is mediated essentially through the so called P-box, a short stretch of amino acids located in the N-terminal zinc finger in the DNA binding domain (DBD) (Ref. 1 and references therein). Amino acids in the P-box have been demonstrated to make direct contacts with bases in the DNA response elements, thus participating in dictating the DNA binding specificity (23). The P-box of ERα (EGCKA) differs from the P-box of other members of the steroid receptor subgroup, such as glucocorticoid receptor and progesterone receptor, with the result that ERα recognizes DNA elements contrasting from the sequences recognized by GR and PR. The consensus ERE consists of a palindromic repeat of the core sequence AGGTCA spaced by three nucleotides (24). The high degree of conservation in the DBD of the α and β ER subtypes (~96%, Fig. 1) and the absolute identity of the P-box sequences strongly suggest a shared DNA recognition specificity between the two ER subtypes. We consequently performed DNA binding studies with mERβ using radiolabeled consensus ERE oligonucleotides.

Mouse ERβ and human ERα protein were synthesized *in vitro* in a rabbit reticulocyte lysate (RRL) system before incubation with E_2 and a radiolabeled double-stranded ERE oligonucleotide. The resulting DNA-protein complexes were analyzed by electrophoretic gel mobility shift assay (Fig. 2). ERβ binds to the wild type consensus ERE both in the absence or presence of E_2 (lanes 2 and 3), but not to an ERE mutated in both half-sites (Fig. 2, lanes 7 and 8). This coincides with the binding specificity of ERα (Fig. 2, lanes 4 and 5 and lanes 9 and 10, respectively). In contrast to results obtained in a recent study by Tremblay and co-workers (25), we did not observe a reduced affinity for the ERE by ERβ when equal amounts of ERα and ERβ protein were used (as quantitated by [^{35}S]methionine labeling). The reason for this discrepancy is unclear.

ERβ Binds to th ERE as a Homodim r

Efficient DNA binding and transactivation function of nuclear receptors are often dependent on the forma-

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mERa MTMTLHTKASGMALLHQIQGNELEPLNRPQLKMPMERALGEVYVDNSKPTVFNYPEGAAYE  61
rERa MTMTLHTKASGMALLHQIQGNELEPLNRPQLKMPMERALGEVYVDNSKPAVFNYPEGAAYE  61

mERb                     MAFYSPAVMNYSVPSSTGNLEGGPVRQTASPNVLWPTSGHL  41
rERb                     .T...........G..S..D.....LST............  41
mERa FNAAAAAAAA-ASAPVYGQSGIA.G.GSEAAAFSANSLGAFPQLNSVSP..LM.LHPPPQ. 121
rERa FNAAAAAAAAGASAPVYGQSSIT.G.GSEAAAFGANSLGAFPQLNSVSP..LM.LHPPP.V 122

mERb SP-LATHCQSSLLYAEPQKSPWC----EARSLEHTLPVNR-ETLKRKLGGSGCASPVTSPS  96
rERb ..-...................----.............-.......S..S........N  96
mERa ..F.HP.G.QVPY.L.NEP.AYAVRDTGPPAFYRSNSD..RQNGRER.SS.NEKGNMIME. 182
rERa ..F.HP.GHQVPY.L.NEP.AYAVRDTGPPAFYRSNSD..RQNGRER.SS.SEKGNMIME. 183
                  CI                                 CII
mERb TKRDAHFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKNCQ 157
rERb A.......P.................................................S.. 157
mERa A.-ETRY....N..............................M...............S.. 242
rERa A.-ETRY....N..............................M...............S.. 243

mERb ACRLRKCYEVGMVKCGSRRERCGYRIVR--RQRSASEQVHCLNKAKRTSGHTPRVKELLLN 216
rERb ............................--....S.......S....NG..A........S 216
mERa ............M.G.I.KD.R.G.MLKHK...DDL.GRNEMGASGDMRAANLWPSP.VIK 303
rERa ............M.G.I.KD.R.G.MLKHK...DDL.GRNEMGTSGDMRAANLWPSP.VIK 304

mERb ---------SLSPEQLVLTLLEAEPPNVLVS-RPSMPFTEASMMMSLTKLADKELVHMIGW 267
rERb ---------T.....................-............................. 267
mERa HTKKNSPAL..TAD.M.SA..D....MIYSEYD..R..S.....GL..N...R......N. 364
rERa HTKKNSPAL..TAD.M.SA..D....LIYSEYD..R..S.....GL..N...R......N. 365

mERb AKKIPGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGI 328
rERb ............................................................. 328
mERa ..RV...GD.N.H...H...CA.L.I..I..V...ME.....L...N.L...NQ......M 425
rERa ..RV...GD.N.H...H...CA.L.I..I..V...ME.....L...N.L...NQ......M 426

mERb LEIFDMLLATTARFRELKLQHKEYLCVKAMILLNSSMYH-LATASQEAESSRKLTHLLNAV 388
rERb .............S.........................P-..S.N............... 388
mERa V...........SS...MMN..GE.FV.L.SI.......GV.TF.SSTLKSL.EKDHIHRV.DKI 486
rERa V...........SS...MMN..GE.FV.L.SI.......GV.TF.SSTLKSL.EKDHIHRV.DKI 487

mERb TDALVWVISKSRISSQQQSVRLANLLMLLSHVRHISNKGMEHLLSMKCKNVVPVYDLLLEM 449
rERb ......E...A..G............................................... 449
mERa ..T.IHLMA.AGLTL....HR...Q..LI....I..M.........YN.........L.... 547
rERa N.T.IHLMA.AGLTL....HR...Q..LI....I..M.........YN.........L.... 548

mERb LNAHTLRG--YKSSISGSGCCSTE-DSKSKEGSQNLQSQ                       485
rERb .........-........E.S...-...N..S.......                       485
mERa .D...R.HAPASRMGVPPEEPSQ.QLATT.STSAHS..TYYIPPEAEGFPNTI         599
rERa .D...R.HAPASRMGVPPEEPSQSQLTTT.STSAHS..TYYIPPEAEGFPNTI         600
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Fig. 1. The Putative Deduced Amino Acid Sequence of the Mouse ERβ (mERβ) Aligned to the Rat ERβ (rERβ) and Mouse and Rat ERα (mERα and rERα) Deduced Amino Acid Sequences, Respectively

The DBD is *boxed*, the two zinc fingers *over-lined* (CI, CII), and the ligand-binding domain is *boxed* and *shaded*.

tion of dimers. Members of the steroid receptor subgroup have been shown to interact with DNA predominantly as homodimers, whereas the receptors for retinoic acid, thyroid hormone, and vitamin D, as well as several orphan nuclear receptors, form heterodimeric complexes with the ubiquitous partner RXR (retinoid X receptor) (26). Importantly, some orphan nuclear receptors have been demonstrated to bind to DNA as monomers (27). To establish whether mERβ binds to DNA as a homodimer or as a monomer, we generated a deletion mutant construct in which the 91 most N-terminal amino acids of the A/B domain of ERβ were removed (Fig. 3A). This region of steroid receptors has not been shown to participate in DNA recognition, and we therefore anticipated that the truncated mERβ would be able to bind to DNA as efficiently as the wild type receptor. The mERβ construct was also tagged with a nine-amino acid HA1-epitope, which is recognized by the monoclonal 12CA5 antibody (28). The truncated epitope-tagged mERβ (ERβ-TAG) in electrophoretic mobility shift assays gave rise to a DNA-protein complex clearly distinguishable from the complex formed by the wild type ERβ (ERβ-wt), (Fig. 3B). After mixed synthesis of ERβ-TAG and ERβ-wt in

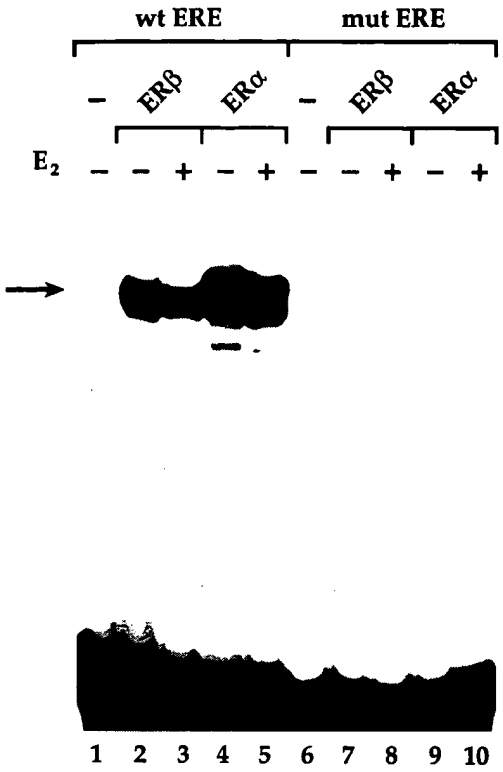

Fig. 2. *In Vitro* Synthesized Mouse ERβ Binds to an ERE

^{32}P-labeled DNA fragments corresponding to a consensus ERE (lanes 1–5) or a derivative ERE mutated in both half-sites (lanes 6–10) were incubated with unprogrammed lysate (lanes 1 and 6) or ERβ (lanes 2–3 and 7–8) or ERα (lanes 4–5 and 9–10) synthesized in the RRL system. DNA-protein complexes were fractionated on a nondenaturing acrylamide gel. The gel was dried and autoradiographed at −70 C.

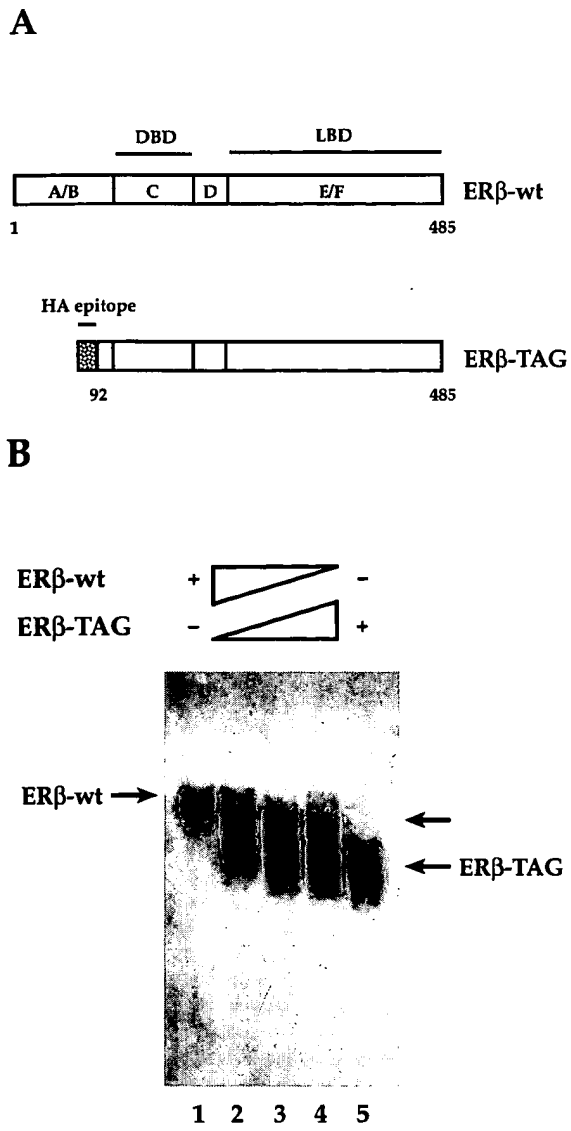

Fig. 3. ERβ Binds to the ERE as a Dimer

A, Schematic presentation of the wild type ERβ (ERβ-wt) and the truncated receptor (ERβ-TAG). The DBD and the LBD are *overlined* in the wild type receptor. The inserted hemaglutenine (HA) epitope of the ERβ-TAG is indicated (*dotted*) and *overlined*. B, ^{32}P-labeled consensus ERE was incubated with wild type ERβ alone (ERβ-wt, lane 1), or ERβ-TAG alone (lane 5), or both proteins together in 3:1, 1:1, and 1:3 ratios (lanes 2, 3, and 4, respectively) after synthesis in the RRL system. DNA-protein complexes were analyzed as described in the legend to Fig. 2. *Arrows* point to complexes formed by ERβ-wt and ERβ-TAG as indicated. The *arrow without label* indicates the band with intermediate migration, which represents the heterodimer of wild type and truncated receptors (lanes 2–4).

the RRL system, a third complex of intermediate mobility was visible (Fig. 3B, lanes 2–4) representing the dimer between wtERβ and ERβ-TAG. This experiment clearly shows that ERβ binds to DNA as a homodimer, similar to the binding of ERα (Refs. 1 and 2 and references therein).

Coexpression of ERβ and ERα Does Not Inhibit ERα-Stimulated Activity of an ERE-Reporter Gene Construct

Having established the ability of mERβ to bind to an ERE, we proceeded to characterize the capacity of mERβ to activate transcription of an ERE-containing reporter gene in transient cotransfections of mammalian cells. We also wanted to study whether cotransfection of both ER subtypes would alter the activity of the ERE reporter construct. Human fetal kidney 293 cells were cotransfected with a luciferase enzyme reporter gene construct containing two copies of a consensus ERE in front of a thymidine kinase promoter, together with human ERα- or mERβ-expressing plasmids, or both. As shown in Fig. 4, the E_2-stimulated activity of the reporter gene construct by mERβ was lower when compared with the activity obtained with ERα when studied under the same transfection conditions. The 2-fold lower E_2-stimulated activity was not

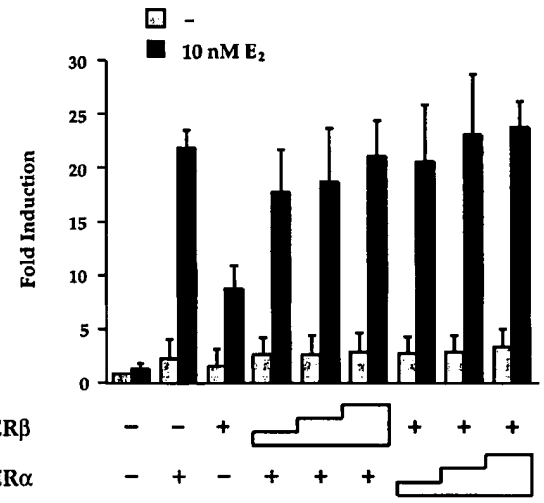

Fig. 4. ERβ and ERα Activate Transcription from an ERE-Containing Reporter Construct

Human fetal kidney 293 cells were transfected with a luciferase reporter construct containing a tandem ERE and ERβ or ERα expression plasmids as indicated (described in *Materials and Methods*). Cells were treated with vehicle (–, *gray bars*) or 10 nM 17β-estradiol (10 nM E_2, *black bars*). The results are presented as fold induction over values obtained from cells transfected with only the luciferase reporter and treated with vehicle, which were arbitrarily set to 1. Values represent the mean ± SD of three independent experiments.

due to squelching of mERβ, since the reporter activity was dose-dependent with regard to the amount of cotransfected mERβ-expression plasmid (data not shown). Although ERβ and ERα are highly homologous in the DBD and in parts of the ligand-binding domain (LBD), there remain substantial differences, particularly in the N-terminal A/B domain (Fig. 1). This domain contains the AF-1 transcriptional activity function of ERα (29) and may have a similar function in ERβ. The diverging A/B domains and/or dissimilarities in the LBD of ERβ and ERα may result in differences in maximal transactivational activity of both ER subtypes. The slightly lower maximal transactivational activity of ERβ compared with ERα has also been observed by other investigators (25, 30). When ERα and ERβ were cotransfected, however, the reporter activity did not change significantly, when compared with the activity observed with ERα alone (Fig. 4). Based on these observations we conclude that ERβ does not repress the activity of ERα. Furthermore, if the two receptors were competing as homodimers for the ERE-binding sites, higher concentrations of ERβ would be expected to result in a decrease in reporter activity, toward the activity pattern of ERβ alone. Since no sign of such a competition was observed, we speculated that an interaction was taking place between the two ER subtypes (although we cannot rule out that ERα alone is responsible for the transcriptional activity of the reporter gene). To be able to monitor a possible interaction between ERβ and ERα, we used a mammalian cell two-hybrid system.

ERβ Interacts with ERα *in Vivo* and *in Vitro*

The two-hybrid system provides a powerful technique for studying potential interactions between two proteins within a cell. The principle is, in short, to fuse protein A to an autonomous DBD and protein B to a strong transactivation domain. The hybrid protein constructs are cotransfected into cells together with a reporter gene construct containing the cognate response element. The activity of the reporter gene will depend on an interaction between the fusion proteins, which will direct the transactivation domain to the promoter. The system has been widely used in yeast, but is also applicable in mammalian cells. For our studies we chose to use the DBD of the yeast protein Gal4 and the transactivation domain of the viral factor VP16. The Gal4-DBD was fused to the full-length mERα, and the VP16 transactivation domain was coupled to full-length mERα and mERβ, respectively (Fig. 5A). The chimera constructs were then cotransfected together with a Gal4-luciferase reporter construct into COS 7 cells. Comparisons were made between cells with or without E_2 incubation. Reporter activity remained low when cells were transfected with the vectors expressing only the Gal4-DBD or the VP16 transactivation domain, or when either of these constructs was transfected together with any of the hybrid constructs (Fig. 5B). In contrast, when the Gal4-mERα construct was transfected together with the VP16-mERα construct or the VP16-mERβ construct, the activity of the reporter was induced approximately 3- to 4-fold, respectively (Fig. 5B, two most *right-hand* panels, *white bars*), compared with reporter transfected alone, indicating an interaction between the chimeric proteins. In the presence of E_2 the induction levels rose to about 18-fold (Fig. 5B, two most *right-hand* panels, *black bars*). These results clearly demonstrate an interaction between the mERβ and mERα proteins *in vivo*, suggesting that the transcriptional activity observed during coexpression of ERα and ERβ (Fig. 4) is, at least in part, due to the formation of ERα/ERβ heterodimers. The rise in reporter activity observed, especially in the presence of E_2 (~5-fold), with the Gal4-mERα hybrid and the original VP-16 construct (Fig. 5B, *second* panel) is in all likelihood due to the ligand-inducible transactivation function of ERα itself, when directed to the promoter of the Gal4 reporter gene construct by binding via the Gal4 DBD.

We also performed glutathione *S*-transferase (GST) pulldown experiments with [^{35}S]methionine-labeled ERα and a GST-mERβ fusion protein, in order to detect a direct interaction between the two proteins *in vitro*. As shown in Fig. 5C, ERα could be successfully coprecipitated with the GST-mERβ fusion protein but not with the GST alone (compare lane 2 to lane 3, lane 1 = input of ERα, 20%), demonstrating a direct interaction between both ER subtypes.

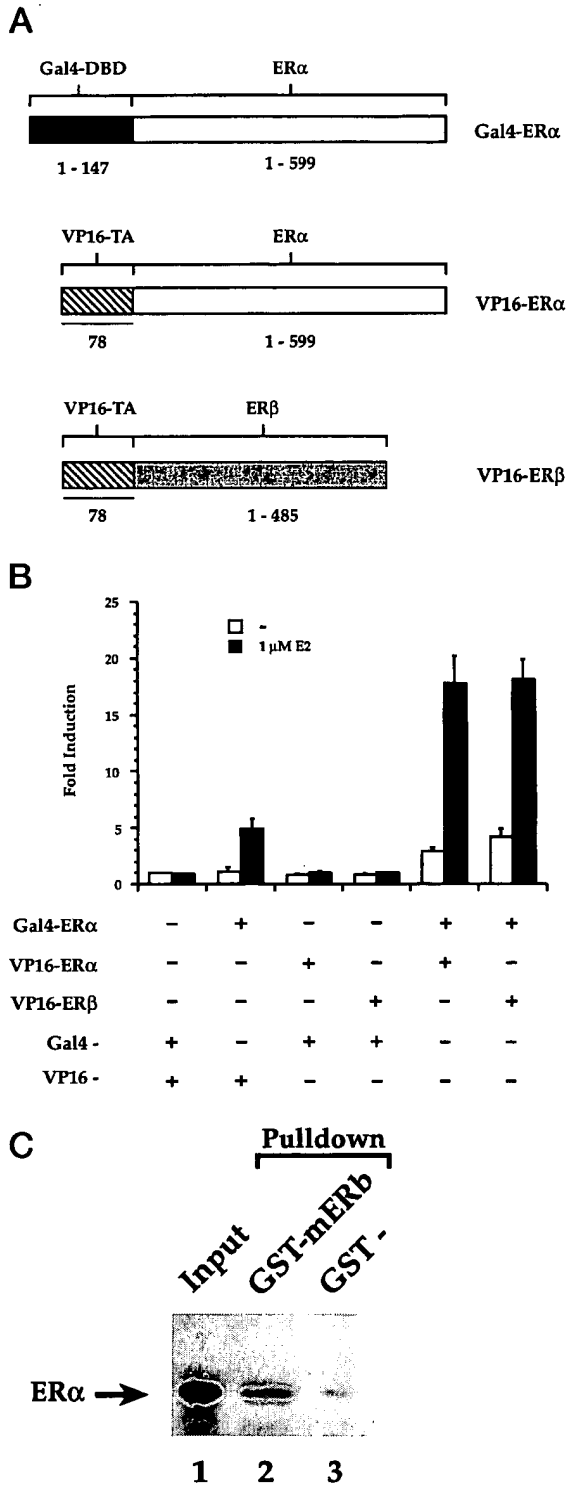

Fig. 5. ERβ Interacts with ERα in a Mammalian Two-Hybrid Protein System

A, Schematic description of the fusion proteins used in the two-hybrid assay. The Gal4-DBD was fused to the full-length ERα, creating Gal4-ERα, and the transactivating domain of VP16 (VP16-TA) was linked to full-length ERα and ERβ, creating VP16-ERα and VP16-ERβ, respectively. B, COS-7 cells were transfected with a luciferase reporter plasmid containing Gal4-binding sites and expression plasmids for Gal4 (Gal4-), VP16 (VP16-), Gal4-ERα, VP16-ERα, and VP16-ERβ as indicated. The cells were treated with vehicle (−, *white bars*) or 1 μM 17β-estradiol (1 μM E_2, *black bars*). Data are presented as fold induction and represent the mean ± SD of three separate experiments performed in duplicate. The values obtained from cells transfected with reporter alone and treated with vehicle were arbitrarily set to 1. C, ERα was labeled with [^{35}S]methionine by translation in RRL and incubated with GST-mERβ or GST-protein. Samples were subsequently incubated with GST-Sepharose, washed, eluted in SDS buffer, and separated on 10% SDS-PAGE gels.

ERβ and ERα Form DNA-Binding Heterodimers

The results from the two-hybrid assay and from the pulldown experiment suggested that ERα and ERβ are able to form heterodimers. In combination with the results from the cotransfection experiments (Fig. 4), it appeared likely that the putative ERβ/ERα heterodimer would be able to bind to an ERE.

We performed electrophoretic mobility shift assay with *in vitro* synthesized ERα and ERβ to examine this possibility. Because the wild type ERβ migrates closely with ERα on native gels (see Fig. 2), we decided to use the truncated ERβ-TAG (described above and in *Materials and Methods*) in order to identify DNA-protein complexes. ERβ-TAG and ERα were synthesized *in vitro* and then mixed at increasing and decreasing amounts, respectively, incubated on ice, followed by incubation with the ^{32}P-labeled ERE. An ERE-protein complex of intermediate mobility was formed in samples in which the two ER subtypes were coincubated (Fig. 6A, lanes 2–5), probably representing a heterodimeric complex between ERβ-TAG and ERα. This putative heterodimerization was also evident when full-length wt ERβ was used instead of ERβ-TAG, but the complexes were not as easily distinguished (not shown).

To confirm the presence of both ER subtypes in the heterodimeric complex, the DNA-binding assay was also performed in the presence of an ERα antibody and the 12CA5 antibody. Figure 6B shows the result of incubation with the monoclonal ERα antibody 1D5. The ERα homodimer is efficiently supershifted with this antibody as expected (Fig. 6B, lane 3 compared with lane 2), whereas the ERβ homodimer is not affected (lanes 9 and 10). The intermediate complex formed in the presence of both ERα and ERβ-TAG is also supershifted with the ERα antibody (Fig. 6B, lanes 5–8 compared with lane 4), thus demonstrating the presence of ERα within the heterodimeric complex. In Fig. 6C essentially the same experiment has been repeated using the 12CA5 monoclonal antibody directed against the HA-epitope of mERβ-TAG. The 12CA5 antibody successfully interacts with both the ERβ homodimer and the intermediate complex previously shown to contain also ERα (Fig. 6C, lanes 10 and 4–7). The 12CA5 antibody does not cross-react with

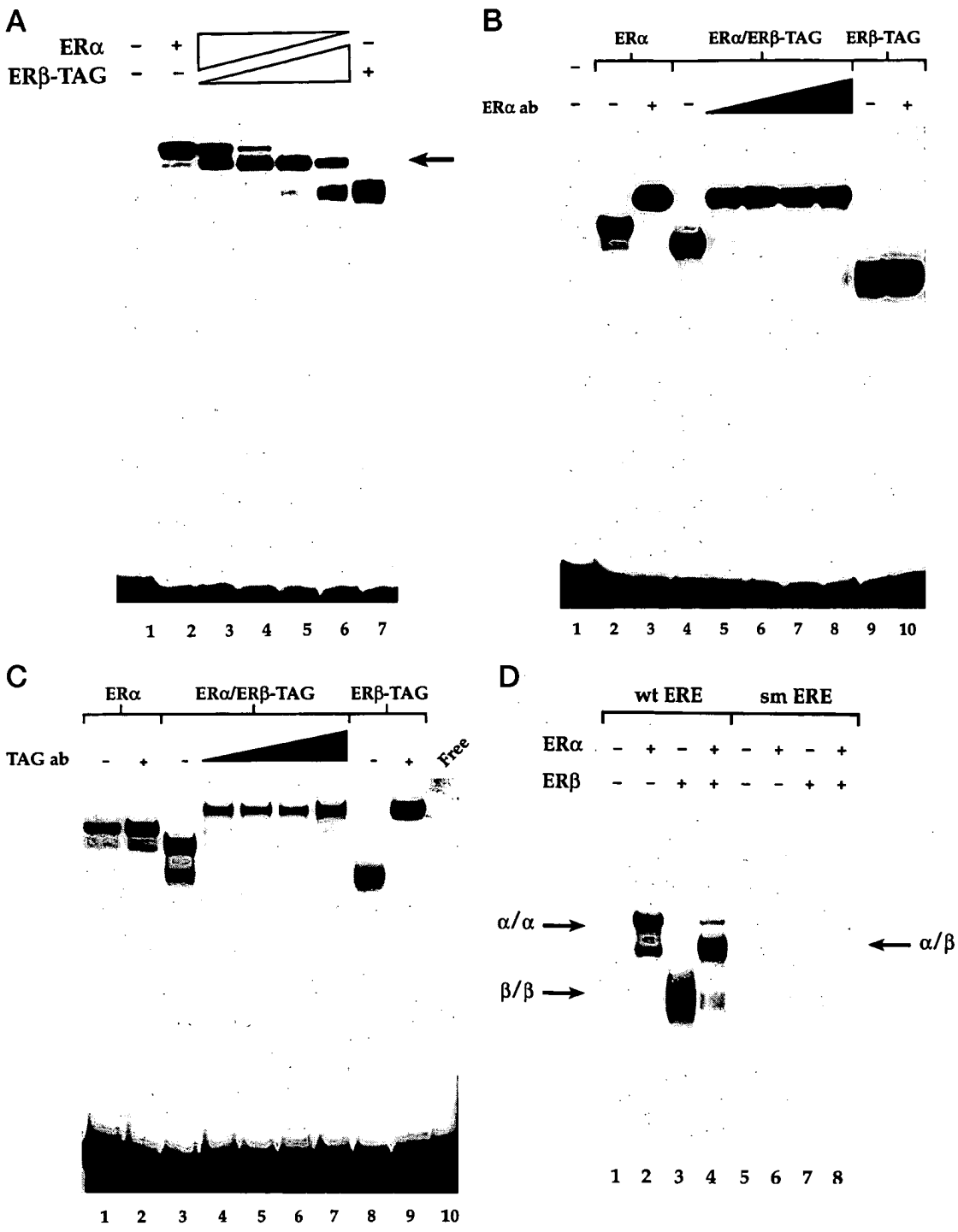

Fig. 6. ERβ Forms DNA-Binding Heterodimers with ERα

A, ERα or ERβ-TAG alone (lanes 1 and 7, respectively) or together as indicated (lanes 2–6) were incubated with a ^{32}P-labeled consensus ERE, and DNA-protein complexes were separated on a nondenaturing acrylamide gel. The *arrow* indicates the intermediate complex formed in the presence of both ERα and ERβ. B, ERα or ERβ-TAG alone or together were incubated with a mouse monoclonal ERα-specific antibody (ERα ab, lanes 3, 5–8, and 10) or with no antibody (lanes 2 and 9) together with ^{32}P-labeled ERE. Complexes were analyzed as above. C, Essentially the same experiment as in B except that the 12CA5-TAG antibody recognizing the epitope-tagged ERβ (TAG ab, lanes 2, 4–7 and 9), or no antibody was used (lanes 1, 3, and 8). D, ^{32}P-labeled DNA fragments corresponding to either a consensus ERE (lanes 1–4) or a derivative ERE mutated in one of the half-sites (lanes 5–8) were incubated with unprogrammed lysate (lanes 1 and 5) or ERα (lanes 2 and 6) or ERβ (lanes 3 and 7) or a mixture of both ERα and ERβ (lanes 4 and 8). DNA-protein complexes were analyzed as described above.

the ERα homodimer (lane 2). These results clearly demonstrate that the intermediate complex that is formed when ERα and ERβ are coincubated contains both receptors and is a true heterodimeric complex.

To determine whether both partners of the heterodimer participate in DNA binding, experiments were performed with an ERE mutated in one of the half-sites at a position previously demonstrated to be crucial for efficient binding by the ERα protein (31). In crystallographic studies of the DBD of ERα bound to the ERE (31), it was established that a mutation in one of the half-sites resulted in reduced cooperativity in binding to the second half-site, due to lack of proper interaction between the dimer interfaces present in the DBD. Binding by ERα to such a mutated ERE was therefore less efficient. We found that ERβ did not bind to this mutated ERE in analogy to the ERα (Fig. 6D, lanes 7 and 6, respectively). In addition, no protein-DNA complex was formed with the heterodimer (lane 8), indicating that cooperativity in DNA binding is also required for efficient DNA binding by ERβ/ERα heterodimers.

Furthermore, the ERβ as well as ERα were unable to bind to oligonucleotides containing direct repeats of the core sequence AGGTCA spaced by one or four nucleotides (DR1 or DR4), irrespective of the presence or absence of retinoid X receptor (data not shown).

Our findings on ERα/ERβ heterodimerization and the recent demonstration of GR/MR heterodimerization (32–33) challenge the commonly held view that steroid receptors form only homodimers. Previous biochemical and structural evidence indicated that steroid receptors form homodimers through a dimerization interface within their zinc finger DNA binding domain, and a generally much stronger dimerization interface within the ligand binding domain (Refs. 1 and 22 and references therein). Further studies will be required to localize the dimerization interfaces involved in the formation of ERα/ERβ heterodimers.

The rat tissue distribution and/or relative level of ERα and ERβ mRNA seems to be quite different; that is, moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ERα and prostate, ovary, uterus, lung, bladder, brain, and testis for ERβ (17). This may imply that in testis and ovary both subtypes are expressed to some extent. In the mouse, both ER mRNAs can be found in ovary and uterus (not shown). In the rat, hypothalamus ERα and ERβ are coexpressed in certain regions, most likely in the same neurons (34). The coexpression of ERα and ERβ in the same tissue and/or cells suggests the interesting possibility that ERα and ERβ proteins may interact with each other. In this study we have indeed shown that the two ER subtypes have the ability to form heterodimers. The discovery of an ERβ protein and the ability of ERα and ERβ to form heterodimers strongly suggest the existence of two previously unrecognized pathways of estrogen signaling: via ERβ homodimers in cells exclusively expressing this subtype and via ERα/ERβ heterodimers in cells expressing both subtypes (Fig. 7). The ERβ homodimers and the ERα/ERβ heterodimers may possibly interact with novel response elements, different from the known EREs. By such a mechanism the physiological regulatory potential of estrogenic hormones may be greatly expanded. Different target tissues may respond differently to the same hormonal stimulus due to alternative composition of receptors. Varying ratios of ERα and ERβ in different cells, resulting in different populations of homo- and heterodimers, could constitute a hitherto unrecognized mechanism involved in the tissue- and cell type-specific effects of estrogens and certain antiestrogens (19, 20).

Future studies will be required to determine the physiological significance of the existence of more than one ER protein.

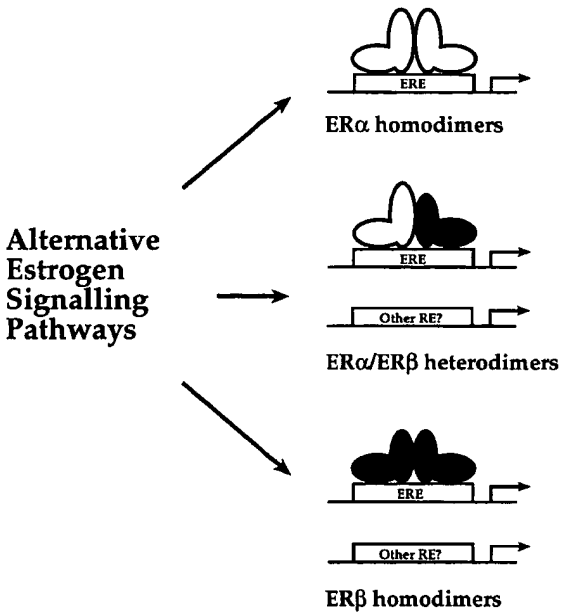

Fig. 7. Alternative Estrogen-Signaling Pathways

The existence of two ER subtypes and their ability to form DNA-binding heterodimers suggests the existence of three potential alternative pathways of estrogen signaling. In cells expressing only the ERα or ERβ subtype, homodimers of either subtype can interact with response elements in target gene promoters and influence transcription levels. In cells expressing both subtypes, heterodimers can be formed depending on the ratio of the subtypes. Although we have shown that ERβ homodimers and ERα/ERβ-heterodimers interact with consensus EREs, it cannot be excluded at this stage that unique response elements exist within the context of target gene promoters that interact preferentially with the ERβ homodimer or the ERα/ERβ-heterodimer. In that way, the regulatory potential of the liganded ER protein could be greatly expanded. An open question in this regard is the existence of estrogen target genes that are exclusively regulated by either of the homodimers or the heterodimer.

MATERIALS AND METHODS

Cloning of Mouse ERβ cDNA

Total RNA from ovaries dissected from 5-week-old mice was prepared as described (35). Complementary DNA was synthesized using Super-Script reverse transcriptase (GIBCO BRL, Paisley, Scotland) as described previously (36) using 1 μg of total RNA. PCR amplification of the cDNA was carried out with 35 cycles of repeated denaturation for 15 sec at 95 C, 15 sec of annealing at 57 C, and 60 sec of extension at 72 C with *Taq*-polymerase (Pharmacia, Uppsala, Sweden) under the conditions described by the manufacturer with 1:20 of synthesized cDNA and oligonucleotides Erbkg3 5′-ATGAGTATTCAGCCATGGCATTCTACAG and Erbkg4 5′-CAGGCCTGGCCATCACTGAGACTG, which were constructed to encompass the entire coding region of rat ERβ cDNA. To facilitate subsequent subcloning, an *Nco*I restriction enzyme recognition site was introduced over the start codon (bases −2 to +4). The PCR product was visualised on a 1% agarose gel, revealing a single band of approximately 1450 bp, corresponding in size to the rat ERβ open reading frame. The DNA band was excised and the DNA was purified using the QIAEX gel purification kit (QIAGEN, Hilden, Germany). The resulting DNA was phosphorylated with T4 polynucleotide kinase (Amersham, Solna, Sweden) and cloned into the T-overhang vector pTKS (37). The insert of pTKS-mERβ was sequenced by the dideoxy method (38) with T7 DNA-polymerase (Pharmacia).

Plasmid Constructs

For *in vitro* transcription/translation in RRL, pTKS-mERβ was digested with *Nco*I and *Eco*RI, which yielded a fragment encompassing the entire coding sequence (cds), which was inserted Sp6-sense into *Nco*I/*Eco*RI-digested pSP72 (Promega, Madison, WI), thus generating pSP72-mERβ. pSP72-mERβ-TAG was made by replacement of nucleotides 1–273 of the mERβ cds with oligonucleotides 5′-CATGGGCTACCCCTACGACGTGCCCGACTACGCCGTGAACA and 5′-CTAGTGTTCACGGCGTAGTCGGGCACGTCGTAGGGGTAGCC, which encode the HA1 epitope recognized by the 12CA5 monoclonal antibody. The plasmid pSP72-hERα has been described elsewhere (36). For transfections of mammalian cells the *Xho*I/*Bgl*II-fragment from pSP72-mERβ was inserted into the pSG5 expression vector (Stratagene, La Jolla, CA) digested with *Eco*RI/*Bgl*II; the *Xho*I-site of the mERβ-fragment and the *Eco*RI-site of pSG5 were filled in with Klenow fragment to allow blunt-ended ligation. The vector pSG5-hERα was made by excising hERα cDNA from pSP72-hERα with *Eco*RI and *Sac*I and inserting it into pSG5 digested with *Eco*RI and *Bgl*II. To enable this ligation, the *Sac*I site was filled in with T4 DNA-polymerase and *Bgl*II with Klenow fragment. The reporter construct 2xERE-TK-Luc was constructed by subcloning of a tandem ERE (39) with *Xho*I overhangs into the *Sal*I site of the p19-TK-Luc reporter plasmid (40). The Gal4-mERα and VP16-mERα two-hybrid constructs were made through PCR amplification of the plasmid MOR101 (containing the mouse ERα cDNA) (41) to introduce a *Kpn*I site upstream of the start codon of the mERα, with the use of oligonucleotides mER-ATG (5′-GCCAGGTACCATGGCCATGACC) and mER-*Eag*I (5′-CCCAGGCTGTTGGCACTGAAGGC). The 275-bp long PCR product was cut with *Kpn*I and *Eag*I, MOR101 was digested with *Eag*I (nucleotide 460 in the mouse ERα cDNA) and *Bam*HI 3′ of the mERα cDNA), and both fragments were subcloned into the *Kpn*I and *Bam*HI sites of pCMX-Gal4 or pCMX-VP16 (42). VP16-ERβ was made by in-frame insertion of the *Nco*I/*Eco*RI fragment of pSP72-mERβ into the *Eco*RV/*Eco*RI sites of pCMX-VP16, and the *Nco*I site of mERβ was filled in with Klenow fragment to enable the ligation. The Gal4-luciferase reporter construct used in the two-hybrid assay has been described elsewhere (43). The pGST-mERβ was generated by in-frame ligation of the *Nco*I/*Eco*RI fragment from pSP72-mERβ into the corresponding sites of pGST (I. Pongratz and F. Delauney, unpublished data) creating a GST-mERβ fusion that could be translated in the RRL system.

Cell Culture and Transient Transfections

Cells from the human fetal kidney cell line 293 were routinely cultured in a 1:1 mixture of Ham's Nutrient mixture F12 (F12, GIBCO BRL) and DMEM (GIBCO-BRL) supplemented with 7.5% FBS, 0.5% nonessential amino acids (NEA, GIBCO BRL) and 1% PEST (100 U penicillin/ml and 100 μg streptomycin/ml). Cells were seeded in six-well plates 24 h before transfection. Transfections using the Lipofectin (GIBCO BRL) reagent were performed as described by the manufacturer in a serum- and antibiotic-free mixture of 1:1 of F12 and phenol-red free DMEM with 0.75 μg of the 2xERE-TK-Luc reporter and 0.1–0.4 μg of pSG5-ERα or pSG5-ERβ as indicated. The pSG5 vector was used to equalize plasmid concentrations, and 0.1 μg of a placental alkaline phosphatase (AP) expression plasmid (44) was included to control for differences in transfection efficiences. Medium was changed to a phenol red-free mixture of F12 and DMEM containing 7.5% dextran-coated charcoal-treated FBS, 0.5% NEA, and 1% PEST after 24 h. Hormone or vehicle (0.1% ethanol) was added simultaneously. Cells were allowed to stand for 48 h with a renewed change of media and hormone after 24 h. Media were collected for assaying of AP activity. The cells were harvested in 10 mM Tris-HCl/10 mM EDTA/150 mM NaCl and centrifuged for 4 min at 4000 rpm, supernatant was removed, and cell pellets were lysed in Lysis Buffer 2 (Bio-Orbit, Turku, Finland). Luciferase activity was measured using the GenGlow system (Bio Orbit). The results are presented as the mean ± SD of fold induction of three separate transfections performed in duplicate.

COS 7 cells were routinely maintained in DMEM (GIBCO BRL) supplemented with 5% FBS and 1% PEST. For transient transfections, cells were seeded in six-well plates 24 h prior to transfection. Transfections were carried out with Lipofectin reagent in phenol red- free DMEM without serum and antibiotics, using 0.5 μg of the GAL4-luciferase reporter construct and 0.1 μg of each of the two-hybrid expression plasmids as indicated in the legend to Fig. 5B. Expression vector concentrations were kept constant in all transfections by addition of the original pCMX-Gal4 or pCMX-VP16 plasmids, and 0.2 μg of the AP expression vector was included in all transfections as an internal control for transfection efficiency. Cells were left in the Lipofectin-DNA mixture for 24 h after which the medium was changed to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS and 1% PEST. Hormone (1 μM E_2) or vehicle (0.1% ethanol) was added. After 24 h cells were harvested as described previously, and luciferase activity was measured. All samples were normalized against the activity of the AP internal standard. Transfections were carried out in duplicate, and the results are presented as fold induction and represent the mean value ± SD of three separate experiments.

In Vitro Translation, GST Pulldown, and DNA-Binding Assays

For the ERE-binding studies, 1 μg pSP72-mERβ or 1 μg pSP72-hERα was transcribed/translated in the TNT-coupled RRL system (Promega) with Sp6 RNA polymerase, according to the manufacturer's instructions, in the presence of 100 nM 17β-estradiol or vehicle (0.01% ethanol). Five microliters of the lysate were used in each DNA-binding reaction with a ^{32}P end-labeled wild type or double-mutated ERE as indicated in the legend to Fig. 2. Protein-DNA complexes were separated on 5% polyacrylamide/0.25× Tris-borate-EDTA gels at ~10 V/cm, followed by drying and autoradiography at −70 C.

In the homodimerization experiments, increasing amounts of pSP72-mERβ-TAG (0, 0.1, 0.2, 0.3, and 0.4 μg) were

translated in the RRL together with decreasing amounts of pSP72-mERβ (0.4, 0.3, 0.2, 0.1, and 0 μg). Five microliters of programmed lysate were used in each DNA-binding reaction with radiolabeled ERE.

For the heterodimerization studies, 1 μg pSP72-hERα or pSP72-mERβ was translated in RRL. In the experiment of Fig. 6A, 5, 4, 3, 2, 1 or 0 μl of ERα-containing lysate were mixed with 0, 1, 2, 3, 4, or 5 μl of ERβ lysate and incubated 15 min on ice before the DNA-binding reaction with radiolabeled ERE.

Translations were carried out in the same manner for the antibody-upshift experiments, and 4 μl ERα or ERβ lysate, respectively, or a mixture of 2 μl of each was incubated for 15 min on ice. Thereafter, 1.5 μl monoclonal ERα antibody 1D5 (Dako, Carpinteria, CA) or 12CA5 TAG antibody (BAbCOf) were added to the respective homodimers and 0.5, 1, 2, or 3 μl of each antibody were added to the heterodimer reactions. The DNA-binding reaction was started immediately. The single-mutated ERE used in the band shift assay in Fig. 6D has been described previously (36). Four microliters of ERα or ERβ protein containing RRL or a mix of 2 μl of each were used in the DNA-binding assay.

For the GST pulldown experiments, pSP72-hERα was translated in the presence of [^{35}S]methionine in RRL and pGST-mERβ or the original pGST was translated in RRL in the absence of radiolabeled amino acids. Five microliters of ERα-containing lysate were mixed with 5 μl lysate containing GST-mERβ or GST-protein. Samples were incubated for 15 min on ice before 50 μl GST-Sepharose diluted in PBS were added to each sample followed by 30 min of incubation on ice. The Sepharose beads were washed four times in PBS/0.1% Triton X-100, and bound proteins were eluted by incubation in 2× SDS-buffer for 5 min at 100 C. ERα lysate (=20%) was loaded as input together with the eluted samples on a 10% SDS-PAGE and run at 150 V. The gel was immersed in 1 M salicylic acid for 20 min, dried, and autoradiographed at −70 C.

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